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Rapid diagnosis of tuberculosis directly from clinical specimens using a gene chip

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Abstract

The aim of this study was to explore a gene chip capable of detecting the presence of *Mycobacterium tuberculosis* isolates directly in clinical sputum specimens and to compare it with current molecular detection techniques. At first, we selected 13 *M. tuberculosis*-specific target genes to construct a gene chip for rapid diagnosis. Using the membrane array method, we diagnosed *M. tuberculosis* by gene chip directly from 246 sputum specimens from patients suspected of having tuberculosis. Among 80 *M. tuberculosis* complex (MTBC) culture-positive sputum specimens, the MTBC detection rate was 62.5% (50/80) by PCR–restriction fragment length polymorphism (RFLP), 70% (56/80) by acid-fast staining, and 85% (68/80) by the membrane array method. Furthermore, subspecies showed different gene expression patterns in the membrane array. In conclusion, MTBC could be detected directly in sputum by the membrane array method. The rapidity of detection and the capability of differentiating subspecies could make this method useful in the control and prevention of tuberculosis.

Keywords: Gene chip, membrane array methods, rapid diagnosis, sputum, tuberculosis
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Introduction

Globally, *Mycobacterium tuberculosis* remains one of the major causes of death due to a single infectious agent. There are an estimated eight million new cases annually, of which approximately 3.5 million are infectious (smear-positive) [1,2]. The incidence differs among countries, depending on social and economic resources, but the vast majority of tuberculosis (TB) patients live in developing countries [3]. The impact is felt most in the developing countries of Asia and Africa, where 95% of cases and 98% of deaths are attributable to the disease [4]. Rapid and reliable laboratory tests for the detection, identification and determination of the susceptibility of mycobacteria are crucial for TB control.

Detection of *M. tuberculosis* complex (MTBC) generally relies on a combination of *in vitro* cultivation and staining of acid-fast bacilli on unprocessed sputum smears with conventional light microscopy [4]. However, the sensitivity has been variable (range of 20–80%) [5]. Even in well-equipped clinical laboratories, approximately 3 weeks are required for the detection of TB cases using liquid culturing systems [6].

With the advent of molecular biological techniques, there have been significant advances in DNA amplification and hybridization that are helping to rectify existing flaws in the diagnosis of TB. The detection of mycobacterial DNA in clinical samples by PCR is a promising approach for the rapid diagnosis of TB. The PCR targets analysed in those samples include IS*6110* [7].

A PCR–restriction fragment length polymorphism (RFLP) analysis of the *hsp65* gene was used for the routine identification of MTBC [8]. However, although PCR and PCR-RFLP analysis can detect the presence of MTBC, they cannot differentiate *M. tuberculosis* from other MTBC members.

Serological tests have been attempted for decades. It has been difficult to develop an ELISA utilizing a suitable antigen, because TB shares a large number of antigenic proteins with other microorganisms that may or may not be pathogenic. Meta-analyses have convincingly shown that existing commercial antibody-based tests have limited clinical utility [9].

In previous studies, we used a well-established membrane array platform for multi-target detection. With this method [10,11], we analysed MTBC-associated mRNA expression in sputum and compared the consistency and convenience with that of PCR-RFLP. In order to directly identify the presence of *M. tuberculosis*, we selected 13 *M. tuberculosis*-specific target genes (i.e. *hsp65*, *Rv0577*, *TbD1*, *Rv3120*, *Rv2073c*, *Rv1970*, *Rv3875*, *Rv0186*, *Rv0124*, *Rv3347c*, *Rv1510*, *mtp40*, and *mpb83*) within the regions of difference of the bacterium. They were marked as *M. tuberculosis*-specific targets in the literature, in genomic databases, and in Medline [12–20]. We used PCR to construct the *M. tuberculosis* pattern as a reference. Using the membrane array method, we constructed the prototype of the diagnostic chip with 13 *M. tuberculosis*-specific target genes, and diagnosed TB through the examination of newly constructed multiple genetic targets directly from sputum specimens of TB patients. We obtained 246 sputum specimens from patients suspected of having TB, and applied PCR and PCR-RFLP to the genotyping of each of the *Mycobacterium* species. The genetic expression profile obtained with the diagnostic chip was fully matched with the PCR pattern of the candidate genes.

This molecular diagnostic method is not only easy to apply, but also allows for direct detection of bacteria in the sputum specimen. The MTBC detection rate with the membrane array is higher than that obtained with PCR-RFLP. The establishment of the diagnostic gene chip could speed up the whole process of diagnosing TB and may have great potential for clinical application.

Materials and Methods

Sample collection of clinical experiments

A total of 246 clinical sputum specimens were obtained from patients suspected of having TB, at the Kaohsiung

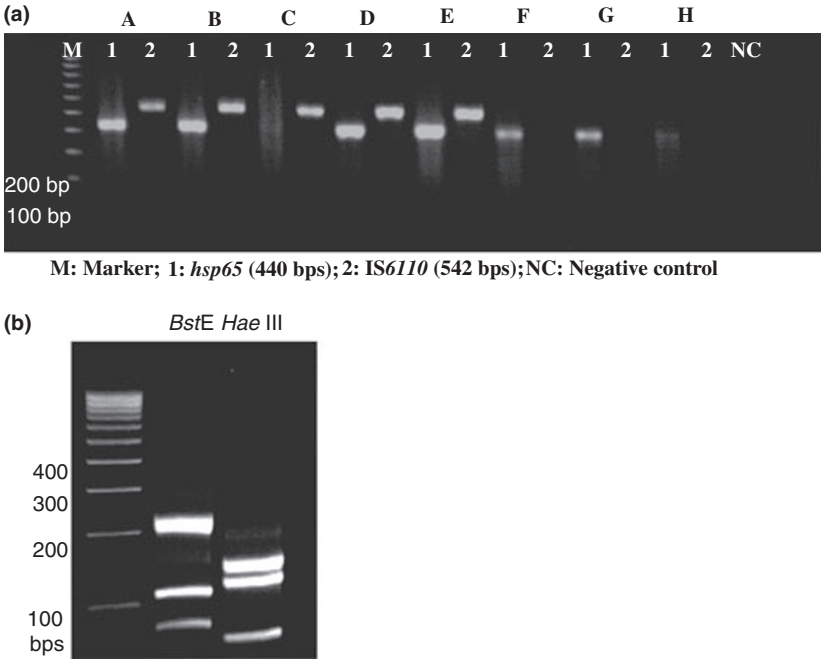


FIG. 1. PCR products from *Mycobacterium* samples obtained with with *hsp65* and IS*6110* probes and results of *Bst*E-generated and *Hae*III-generated restriction fragment length polymorphism (RFLP) patterns. Two sets of oligonucleotide primers were used to amplify target DNA from the multiple-copy *hsp65* gene and IS*6110*. A 440-bp fragment of *hsp65* was amplified by PCR using primers TB11 and TB12. A 542-bp fragment of IS*6110* was amplified by PCR using primers MT1007 and MT1008. The PCR products were analysed by electrophoresis on a 3% agarose gel stained with ethidium bromide, and examined for the presence of 440-bp and 542-bp fragments under UV irradiation. (a) Cases A, B, C, D and E were *hsp65*-positive and IS*6110*-positive; cases F and G were *hsp65*-positive and IS*6110*-negative; case H was *hsp65*-negative and IS*6110*-negative. (b) PCR products were digested with the restriction enzymes *Bst*E and *Hae*III in order to identify *Mycobacterium tuberculosis* complex (MTBC) and non-tuberculous mycobacteria (NTM) on the basis of the RFLP patterns. Cases A, B, C, D and E were confirmed MTBC. Cases F and G were NTM. Case H involved another species.

TABLE 1. Oligonucleotide probes for 13 *Mycobacterium tuberculosis*-specific target genes

Gene	Oligonucleotide
hsp65	AAAGGTGTTGGACTCCTCGACGGTGATGACGCCCTCGTTGCCCACCTTGT
Rv0577	TCCGTGAGCAGTTCGTTCCAGATGAGCGTGCCCGTCTCGTTGACCAACGT
Rv3120	AATACCGCCTCCGTGGGGTCAGCGCACTCGTATTTCGCGTTCCAACGAAT
Rv2073c	GCGTCGTCACTAAGCCGCGTAGTGGTGTTGACCATCGCCACTCACGCTAG
Rv1970	TCTGCTCGGTGCTTGGGTAGGCGCTACCGTGTGACAGCGCAATGAGTGAA
Rv3875	TCCCCTCGTCAAGGAGGGAATGAATGGACGTGACATTTCCCTGGATTGCG
Rv3347c	TGGACGCCCCAACGATCCAGTTGTCGCCGAGCGCATTCACGAACAGCAAC
Rv1510	AGTTTGGTAGTCGGGGCCGAATCCAACACGCAGCAACCAGGGACCGGTAA
Rv0186	GTGCCGGTCGGGTACCGCAATAGTGCCTGTGCCGCCATGGTTTTCATAGT
Rv0124	ACCACAATCTCCGGGGCTACGCTGACAAACGACATCACACACCTCCCCAA
TbD1	GTGTCCAGGACTTGCCGAGGTGTGGCATCCACGTCCAGATAATTGATCGT
mtp40	TGGTCGAATTCGGTGGAGTCGCAAAGTTGAACGCTGAGGTCATGTCGCCA
mpb83	TGCGACACGGGTTTGGTGCTCGAACAACCCGCTAAGAACGCAATCGCGAT

Medical University Hospital, from December 2006 to July 2007. The exclusion criterion was contamination of the specimen. Specimen acquisition and subsequent use were approved by the Institutional Review Board of Kaohsiung Medical University Hospital. After processing for routine mycobacteriology [21], each post-decontamination specimen was divided into two parts: one part (0.5 mL) was used for smear and culture, and the other (0.5 mL) was processed for PCR.

Microscopy and culture

Sputum samples were digested and decontaminated, using the standard protocol, with *N*-acetyl-L-cysteine/2% NaOH (pH 6.8). Phosphate buffer was added, bacteria were concentrated by centrifugation (3000 ***g***) for 15 min [22], and sediments were cultured in triplicate at 37°C and 10% CO_2 on Löwenstein–Jensen slant medium.

The sediment was resuspended in 2 mL of supernatant. All clinical specimens were processed for direct smear examination by fluorescence microscopy after Auramine O/Rhodamine B staining for AFB. Aliquots of the remaining clinical samples and the resuspended sediments of sputum were frozen at −30°C until being processed for PCR.

MTBC strain analysis by PCR and PCR-RFLP

Polymorphic regions of each of the 13 genes (see above) were amplified by multiple PCR with two allele-specific sense (or antisense) primers. Two sets of oligonucleotide primers were used to amplify target DNA from the multiple-copy genes [7]. A 440-bp fragment of *hsp65* was amplified by PCR using primers TB11 and TB12. A 542-bp fragment of IS*6110* was amplified by PCR using the primers MT1007 and MT1008. Products were analysed by electrophoresis on a 3% agarose gel stained with ethidium bromide, and examined for the presence of 440-bp and 542-bp fragments under UV irradiation. PCR products were digested with the restriction enzymes *Bst*E and *Hae*III in order to identify MTBC and non-tuberculous mycobacteria (NTM) on the basis of RFLP patterns (Fig. 1a,b). Thirteen *M. tuberculosis*-specific target genes were analysed by electrophoresis of PCR products.

Preparation of digoxigenin (DIG)-labeled cDNA targets and hybridization

First-strand cDNA targets for hybridization were produced using SuperScript reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA) in the presence of DIG-labeled UTP (Roche Diagnostics GmbH, Penzberg, Germany). After the prehybridization procedure, the gene chips were subjected to hybridization. The arrays were covered with Express Hyb Hybridization Solution (BD Biosciences, Palo Alto, CA, USA) containing DIG-11-UTP-labeled cDNA probes, and then incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) and blocking solution. For hybridization, the arrays were incubated at 42°C for 12 h in a humid chamber. After washing, the arrays were exposed to light. For signal detection, the gene chips were incubated in chromogen solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate for 15 min.

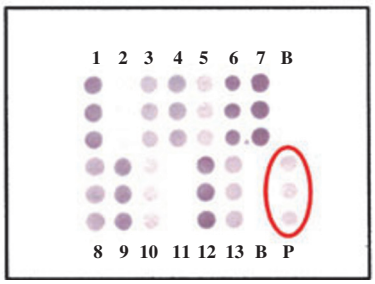
Design of oligonucleotide probes and preparation of the oligonucleotide membrane array

The procedure for the design and preparation of the membrane array was in accordance with our recent work [23]. Visual OMP3 (Oligonucleotide Modeling Platform; DNA Software, Ann Arbor, MN, USA) was used to design probes (Table 1). The probe selection criteria included strong mismatch discrimination, minimal or no secondary structure, signal strength at the assay temperature, and lack of cross-hybridization. Probes were synthesized according to the designed sequences, purified and controlled before being grafted onto the substrates. The newly synthesized oligonucleotides were dissolved in distilled water at a concentration of 20 mM, and applied to a BioJet Plus 3000 nanolitre dispenser system (BioDot, Irvine, CA, USA), which blotted the 13 target gene oligonucleotides (and one housekeeping

(a)

hsp65	Rv0577	Rv3120	Rv2073c	Rv1970	Rv3875	Rv3347c	Blank
hsp65	Rv0577	Rv3120	Rv2073c	Rv1970	Rv3875	Rv3347c	Blank
hsp65	Rv0577	Rv3120	Rv2073c	Rv1970	Rv3875	Rv3347c	Blank
Rv1510	Rv0186	Rv0124	TbD1	*mtp-40*	*mpb83*	Blank	*β*-actin
Rv1510	Rv0186	Rv0124	TbD1	*mtp-40*	*mpb83*	Blank	*β*-actin
Rv1510	Rv0186	Rv0124	TbD1	*mtp-40*	*mpb83*	Blank	*β*-actin

(b)



Lane 1 : *hsp65* ; 2 : Rv0577 ; 3 : Rv3120 ; 4 : Rv2073c ; 5 : Rv1970 ; 6 : Rv3875 ; 7 : Rv3347c ; 8 : Rv1510 ; 9 : Rv0186 ; 10 : Rv0124 ; 11 : TbD1 ; 12 : *mtp40* ; 13 : *mpb83* ; B : Blank ; P : *β*-actin

FIG. 2. Array table of the gene chip and gene expression pattern in a *Mycobacterium tuberculosis* sample. (a) Schematic representation of a membrane array with 13 *M. tuberculosis*-specific target genes, one housekeeping gene (*β-actin*), and a blank control. The positions of 13 *M. tuberculosis*-specific target genes (i.e. *hsp65*, *Rv0577*, *TbD1*, *Rv3120*, *Rv2073c*, *Rv1970*, *Rv3875*, *Rv0186*, *Rv0124*, *Rv3347*, *Rv1510*, *mtp40* and *mpb83*), as well as the correlation between the blank and positive control (*β-actin*) in the nylon membrane, are indicated by spots. The relevant positions are red (*β-actin*), yellow (blank), and blue, green and purple (*M. tuberculosis*-specific target genes). (b) Expression patterns of *M. tuberculosis*-specific target genes in one sample of *M. tuberculosis*; the array results were as follows: *hsp65*, +; *Rv0577*, +; *TbD1*, –; *Rv3120*, +; *Rv2073c*, +; *Rv1970*, +; *Rv3875*, +; *Rv0186*, +; *Rv0124*, +; *Rv3347*, +; *Rv1510*, +; *mtp40*, +; *mpb83*, +; *β-actin*, +.

gene, *β-actin*) sequentially (50 nL per spot; 1.5 mm between spots) onto a Nytranl SuperCharge nylon membrane (Schleicher and Schuell, Dassel, Germany) in triplicate; the oligonucleotides were cross-linked to the membrane using a UV Stratalinker 1800 (Statagene, La Jolla, CA, USA). Each spot contained 20 ng of PCR-amplified DNA derived from sequence-verified cDNA clones. Dimethylsulphoxide was also dispensed onto the membrane as a blank control.

Data analysis of colourimetric membrane

The hybridized arrays were scanned with an Epson Perfection 1670 flatbed scanner (SEIKO EPSON Corp., Nagano-ken, Japan). Subsequent quantitative analysis of the intensity of each spot was performed using AlphaEase FC software (Alpha Innotech Corp., San Leandro, CA, USA). Spots of consistently two fold (or greater) increased intensity with respect to positive control were considered to represent differentially expressed genes. These array analysis tools facilitated the measurement of relative grey levels of objects in a uniformly spaced array, such as in dot blots. A deformable template extracted the gene spots and quantified their expression levels according to the integrated intensity of each spot after background subtraction.

The fold ratio for each gene was calculated as follows: spot intensity ratio = mean intensity of target gene/mean intensity of *β-actin*. Fig. 2a shows a schematic representation of the membrane array with 13 target genes, one housekeeping gene (*β-actin*), and one blank control. The positions of the target genes and the correlation between the blank and positive control (*β-actin*) on the nylon membrane are indicated by spots. A triplicate set of molecular markers were blotted (Fig. 2b). This membrane array was used to detect the gene expression profiles of *M. tuberculosis*-specific target genes in sputum specimens. Sputum samples of 3–5 mL were digested and decontaminated, and 1-mL sediment suspensions were subjected to total RNA extraction and subsequent synthesis of cDNA to be applied to the membrane.

TABLE 2. ***Mycobacterium tuberculosis*-specific target gene expression profiles of individual *M. tuberculosis* complex subspecies observed by membrane array analysis**

	hsp65	*Rv0577*	*TbD1*	*Rv3120*	*Rv2073c*	*Rv1970*	*Rv3875*	*Rv0186*	*Rv0124*	*Rv3347c*	*Rv1510*	*mtp40*	*mpb83*
M. canettii	+	+	+	+	+	+	+	+	+	+	+	+	–
M. tuberculosis	+	+	–	+	+	+	±	+	+	+	+	+	–
M. africanum Ib	+	+	+	+	–	+	+	+	+	±	+	+	–
M. africanum IIb	+	+	±	+	–	–	+	–	–	+	+	+	–
M. microti	+	+	+	+	–	–	–	+	+	–	±	+	–
M. caprae	+	+	+	–	⊥	–	+	+	+	+	+	+	–
M. bovis	+	±	+	–	–	–	+	+	+	+	+	–	+
M. bovis BCG	+	+	+	–	–	⊥	–	+	+	+	–	–	+

+, positive reaction in >90% of total bacterial isolates; ±, positive reaction in 90–60% of total bacterial isolates; ⊥, positive reaction in 10–60% of total bacterial isolates; –, positive reaction in <10% of total bacterial isolates.

Results

The 246 sputum specimens from patients suspected of having TB were analysed. The results concerning IS*6110* and *hsp65* of the *Mycobacterium* isolates were obtained by PCR-RFLP analysis. Following culture, 80 of 246 specimens were found to contain MTBC, 155 were identified as NTM, and 11 were found to be bacteria other than mycobacteria.

According to the results of the membrane array analysis, the MTBC subspecies have individual *M. tuberculosis*-specific target gene expression profiles, as shown in Table 2. For example, *M. tuberculosis* had a >90% positive reaction with respect to *hsp65*, *Rv0577*, *Rv3120*, *Rv2073c*, *Rv1970*, *Rv3875*, *Rv0186*, *Rv0124*, *Rv3347c*, *Rv1510* and *mtp40*, whereas *Mycobacterium bovis* had a >90% positive reaction with respect to *hsp65*, *TbD1*, *Rv3875*, *Rv0186*, *Rv0124*, *Rv3347c*, *Rv1510*, and *mpb83*.

Among these MTBC culture-positive sputum specimens, 56 (70%) were also shown to be smear-positive, PCR-RFLP was positive in 50 (62.5%), and the membrane array was positive in 68 (85%). Of the 24 MTBC culture-positive, stain-negative sputum specimens, 11 (45.8%) tested positive by PCR-RFLP, and 16 (67%) tested positive by the membrane array method. The comparative MTBC detection rates determined using the acid-fast stain, PCR-RFLP method and membrane array method are shown in Table 3.

TABLE 3. Comparison of 80 *Mycobacterium tuberculosis* complex culture-positive sputum specimens by acid-fast stain, PCR–restriction fragment length polymorphism (PCRRFLP), and membrane array

		PCR-RFLP		Membrane array		
		Positive	Negative	Positive	Negative	
Acid-fast stain	Positive	39	17	52	4	
	Negative	11	13	16	8	

Discussion

TB is a bacterial disease caused by MTBC organisms and continues to be a leading cause of death in developing countries. It is transmitted primarily by the nuclei of airborne droplets; however, the diagnosis of TB continues to pose serious problems, mainly because of difficulties in differentiating between patients with active TB and those with healed lesions. Physicians still rely on conventional methods, such as Ziehl–Neelsen staining, fluorochrome staining, and sputum culture. Although the tuberculin test has facilitated diagnosis for more than 85 years, its interpretation is difficult, because sensitization with NTM leads to false-positive test results. Rapid diagnosis and treatment are important for preventing the transmission of the disease.

Microscopy and culture are still the major backbone of laboratory diagnosis of TB. However, a positive smear by Acid-Fast stain (AFS) requires the presence of $\leq 10^4$ AFB per millilitre of sputum, and AFS is unable to identify other acid-fast organisms [24].

Microscopy with material from solid, and often liquid, cultures can be used to make a presumptive diagnosis of TB, although it does not allow a distinction to be made between *M. tuberculosis* and NTM in clinical specimens [25]. The ability to simultaneously assess the smear status and directly distinguish between *M. tuberculosis* and NTM in a clinical specimen has important implications for patient treatment and hospital infection control.

The prompt diagnosis of smear-positive cases is a prerequisite for controlling TB, and several new methods have evolved over the past two decades. The majority of molecular tests have focused on the detection of nucleic acid sequences in both DNA and RNA specific for *M. tuberculosis* by amplification techniques such as PCR. With a smear-

negative specimen, however, a negative PCR result does not exclude the presence of *M. tuberculosis* [7]. In this study, among MTBC culture-positive but smear-negative sputum specimens, PCR-RFLP analysis led to the detection of MTBC in 45.8% of cases, and the membrane array method in up to 67%. Among the culture-positive and sputum smear-positive specimens, PCR gave a 70% identification rate and the membrane array method was positive in 92.8%. Thus, in this study, the membrane array method yielded a higher detection rate than PCR-RFLP for either smear-positive or smear-negative MTBC sputum culture-positive specimens.

Although the virulence and host ranges differ among MTBC members, most conventional molecular techniques are incapable of differentiating among these subspecies, because of the genetic identities of their 16S rRNA gene sequences. Parsons *et al.* [26] reported that the identifications obtained by using the specific deletion profiles correlated well with the original identifications for all MTBC subspecies, except for *Mycobacterium africanum*. MTBC members contain the transposable element IS*6110*, which has become a widely used diagnostic marker in epidemiological studies [27]. However, an IS*6110*-based PCR assay still does not allow differentiation among subspecies of MTBC.

In the modern era of genetics, the day is not far off when chip-based DNA hybridization assays will provide instant diagnosis of mycobacterial infections. In 2002, Brosch *et al.* [14] evaluated the distribution of 20 variable regions resulting from insertion–deletion events in the genomes of the MTBC subspecies. In order to differentiate among the subspecies, we elected 13 specific target genes that were correlated with the regions of difference or marked as MTBC-specific targets in the literature, in genomic databases and in Medline. Using the membrane array method, we found that there were different *M. tuberculosis*-specific target gene expression profiles by examining sputum specimens directly using gene chip-based analysis.

As the membrane array method is capable of detecting multiple gene targets simultaneously, it is more efficient, in terms of time and cost, than PCR [28]. The advantages of membrane array methods are as follows: (i) simultaneous analysis of multiple genes is possible; (ii) a small sample is required, and simultaneous analysis can reduce the consumption of reagents and materials; and (iii) with the use of automated detection software, the biological molecules can be analysed within a short time period. However, the gene chips and fluorescence scanners that are needed to read test results are still expensive; therefore, the popularity remains limited. The membrane array method uses the chemical colourimetric method, which replaces glass chips with the fluorescence of gene chips, and nylon membranes. The entire process of hybridization and colour development takes <12 h in total. The cost (*c.* $50) and technology thresholds are thus reduced significantly, while the original advantages of the gene chip are preserved. Although this technique can significantly reduce the difficulty and cost of experiments (and thereby increase its popularity and application in the medical setting), the reading of colourimetric methods is easily skewed by artefacts.

Application of membrane assays to the control of TB may result in important innovations in diagnosis and treatment. This method has reduced the time to diagnosis, and increased the safety of laboratory technicians and medical technologists. Moreover, the MTBC detection rate of the membrane assay is potentially higher than that of PCR-RFLP assays. Thus, our goal is to develop a sensitive, time-saving and high-throughput active TB chip for the detection of *M. tuberculosis*. In the future, membrane arrays could be adapted to drug susceptibility testing. The differential activities and post-antibiotic effects of various drugs against TB will lead to highly effective, less time-consuming regimens and to directly monitored therapy.

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Transparency Declaration

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The authors affirm the absence of conflicting or dual interests.

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